

# Inactivation of Murine *Usp1* Results in Genomic Instability and a Fanconi Anemia Phenotype

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## SUMMARY

Fanconi anemia (FA) is a human genetic disease characterized by chromosome instability, cancer predisposition, and cellular hypersensitivity to DNA crosslinking agents. The FA pathway regulates the repair of DNA crosslinks. A critical step in this pathway is the monoubiquitination and deubiquitination of FANCD2. Deubiquitination of FANCD2 is mediated by the ubiquitin protease, USP1. Here, we demonstrate that targeted deletion of mouse *Usp1* results in elevated perinatal lethality, male infertility, crosslinker hypersensitivity, and an FA phenotype. *Usp1*<sup>−/−</sup> mouse embryonic fibroblasts had heightened levels of monoubiquitinated Fancd2 in chromatin. *Usp1*<sup>−/−</sup> cells exhibited impaired Fancd2 foci assembly and a defect in homologous recombination repair. Double knockout of *Usp1* and *Fancd2* resulted in a more severe phenotype than either single knockout. Our results indicate that mouse *Usp1* functions downstream in the FA pathway. Deubiquitination is a critical event required for Fancd2 nuclear foci assembly, release from chromatin, and function in DNA repair.

## INTRODUCTION

The Fanconi anemia (FA) pathway mediates DNA repair and promotes normal cellular resistance to DNA crosslinking agents (Wang, 2007). The FA pathway is regulated by 13 FA proteins (FANCA, B, C, D1, D2, E, F, G, I, J, L, M, and N) (Grompe and van de Vrugt, 2007). Eight of the FA proteins are assembled in a nuclear ubiquitin E3 ligase complex (FANCA/B/C/E/F/G/L/M), known as the FA core complex, which monoubiquitinates FANCD2 and FANCI (Dorsman et al., 2007; Sims et al., 2007; Smogorzewska et al., 2007). The monoubiquitinated FANCD2/FANCI complex is targeted to chromatin (Montes De Oca et al., 2004), where it interacts, either directly or indirectly, with additional downstream FA proteins (FANCD1, FANCN, and

FANCI). The downstream FA genes are also breast cancer susceptibility genes (Grompe and van de Vrugt, 2007).

Recent studies have indicated that the FANCD2/FANCI complex is deubiquitinated by the ubiquitin-specific protease, USP1 (Cohn et al., 2007; Huang et al., 2006; Nijman et al., 2005a). *USP1* is not an FA gene per se, since no human FA patients harboring mutations in the *USP1* gene have been identified. Disruption of the *Usp1* gene in chicken cells (DT40) results in crosslinker hypersensitivity, and the chicken *Usp1* and *Fanci* genes are epistatic for crosslink repair (Oestergaard et al., 2007).

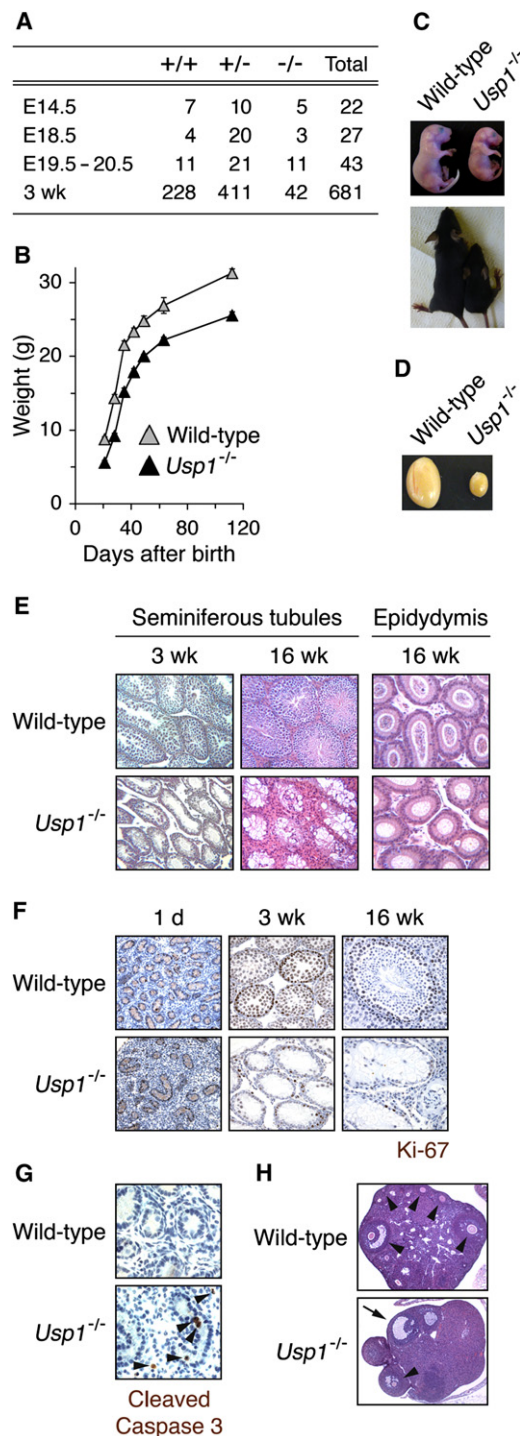
The human genome encodes over 90 deubiquitinating enzymes (DUBs) (Nijman et al., 2005b). In rare cases, disruption of a ubiquitin E3 ligase results in a similar outcome to disruption of a corresponding DUB (Nijman et al., 2005b), suggesting that coupled ubiquitination and deubiquitination may be essential for the function of some pathways.

In the current study, we disrupted the murine *Usp1* gene. Interestingly, *Usp1*<sup>−/−</sup> mice had a strong resemblance to FA mice (small size, infertility, mitomycin C [MMC] hypersensitivity, and chromosome instability). In addition, *Usp1*<sup>−/−</sup> mice exhibited a higher rate of perinatal lethality and absence of male germ cells. Our results indicate that *Usp1* is required for Fancd2 foci assembly and contributes to homologous recombination (HR) repair, suggesting a regulatory role of *Usp1* in the FA pathway.

## RESULTS

### Perinatal Lethality and Growth Retardation in *Usp1*-Null Mutation

To address the physiological role of *Usp1*, we performed a targeted gene deletion of *Usp1* in the mouse (see Figure S1 available online). *Usp1*<sup>+/−</sup> mice were grossly normal and fertile. Murine *Usp1* was detected as two distinct protein products of different size in wild-type mouse embryonic fibroblasts (MEFs), and *Usp1*<sup>+/−</sup> MEFs displayed approximately 2-fold reduction in *Usp1* protein level (Figure S1C). From *Usp1*<sup>+/−</sup> intercrosses, *Usp1*<sup>−/−</sup> mice were obtained at a low frequency (6%, instead of the expected 25%; Figure 1A). When analyzed during gestation (E13.5–E18.5) or after cesarean delivery at E19.5–E20.5, *Usp1*<sup>−/−</sup> embryos, although significantly smaller than their wild-type littermates (Figure 1C and data not shown), were present at the expected Mendelian ratio. Thus, deletion of the *Usp1* gene is



**Figure 1. Perinatal Lethality, Growth Retardation, and Impaired Germ Cell Development in *Usp1*-Deficient Mice**

(A) Viability of *Usp1*-deficient mice at different stages of development. (B) Growth curve of wild-type (gray triangles) and *Usp1*<sup>-/-</sup> (filled triangles) mice. (C) Gross appearances of newborns and 2-week-old wild-type and *Usp1*<sup>-/-</sup> mice. (D) Comparison of testis size from 16-week-old wild-type and *Usp1*<sup>-/-</sup> mice. (E) Sections of wild-type and *Usp1*<sup>-/-</sup> seminiferous tubules and epididymis stained with hematoxylin and eosin. Magnification, 10×.

not embryonic lethal; rather, it led to perinatal lethality, since within 1–2 days after birth, up to 80% of the *Usp1*<sup>-/-</sup> mice were dead. *Usp1*<sup>-/-</sup> pups found dead displayed several abnormalities with incomplete penetrance; these included cyanosis, bilateral hydronephrosis, and hemorrhagic edema. When closely monitored at the moment of delivery, most of the *Usp1*<sup>-/-</sup> pups (75%) became progressively cyanotic and died within 2 hr of birth. Thus, cyanosis may be one of the causes of perinatal lethality in newborn *Usp1*<sup>-/-</sup> mice. The surviving *Usp1*-deficient mice were consistently smaller than their wild-type littermates, at weaning and as adults, suggesting that growth retardation occurred prenatally and persisted into adulthood (Figures 1B and 1C).

#### Gonadal Dysfunction in *Usp1*-Deficient Male Mice

*Usp1*<sup>-/-</sup> male mice were sterile. The *Usp1*<sup>-/-</sup> testis was strikingly smaller than that of wild-type or *Usp1*<sup>+/-</sup> littermates (Figure 1D and data not shown). Histology of adult testes from *Usp1*<sup>-/-</sup> mice revealed that their seminiferous tubules were markedly atrophic and mostly devoid of spermatogenic cells (e.g., spermatogonia, spermatocytes, spermatids, and spermatozoa; Figure 1E). Consistently, the epididymis of *Usp1*<sup>-/-</sup> mice was devoid of spermatozoa (Figure 1E). We examined seminiferous tubules for cellular proliferation by Ki-67 immunostaining (Figure 1F). At 1 day after birth, Ki-67-positive cells were detected in wild-type and *Usp1*<sup>-/-</sup> tubules with similar frequency, and the intensity of Ki-67 staining in *Usp1*<sup>-/-</sup> tubules was even stronger than that of the wild-type tubules. At 3 and 16 weeks, however, cellularity of *Usp1*<sup>-/-</sup> tubules was greatly decreased, and remaining cells were mostly negative for Ki-67 staining (Figure 1F). Immunostaining for cleaved Caspase 3 revealed that *Usp1*<sup>-/-</sup> tubules had a slightly increased frequency of apoptosis 1 day after birth (Figure 1G). However, the majority of *Usp1*<sup>-/-</sup> cells did not express cleaved Caspase 3, suggesting that apoptosis is not the major cause of testicular atrophy in *Usp1*<sup>-/-</sup> mice.

*Usp1*<sup>-/-</sup> female mice showed a reduced fertility as compared with wild-type females. *Usp1*<sup>-/-</sup> ovaries had less oocytes than wild-type ovaries ( $8.5 \pm 0.6$  and  $2.9 \pm 0.3$  per section for wild-type and *Usp1*<sup>-/-</sup> 12-week-old ovaries, respectively; five females each; Figure 1H).

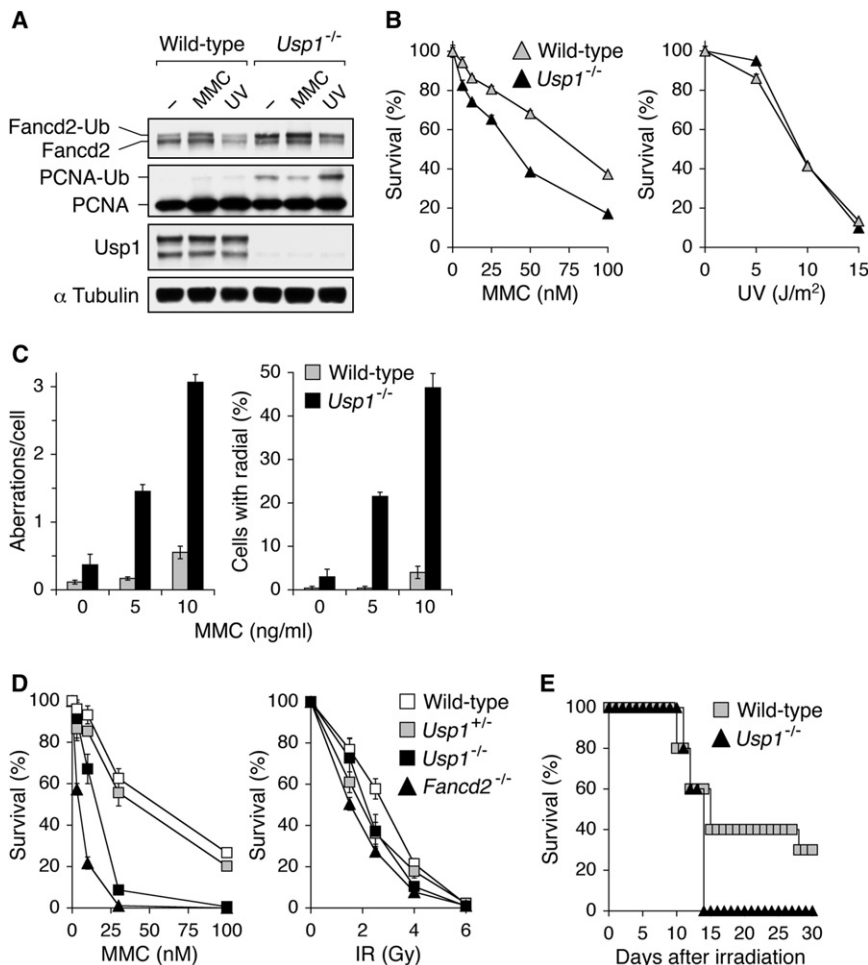
#### MMC Hypersensitivity and Enhanced Fancd2 and PCNA Monoubiquitination in *Usp1*-Deficient Cells

There were no significant differences in cell cycle distribution between wild-type and *Usp1*<sup>-/-</sup> primary MEFs (either asynchronous or after release from synchronization in G0 by serum starvation) (Figures S2A and S2B). As predicted from studies with

(F) Detection of Ki-67 by immunohistochemistry in seminiferous tubules from wild-type and *Usp1*<sup>-/-</sup> mice. Magnification, 40×.

(G) Detection of apoptosis by cleaved Caspase-3 staining in seminiferous tubules from 1-day-old testes. Magnification, 63×.

(H) *Usp1*<sup>-/-</sup> ovaries (lower panel) had reduced numbers of oocytes (arrowhead) compared with wild-type ovaries (upper panel), yet retained the follicle (arrow) from which the oocyte had been released during ovulation. For the quantitative analysis, oocytes were counted from five females of each genotype at 12 weeks, and the number of oocytes was determined for each section (8 sections per each female; total 40 sections). All visible oocytes (including small oocytes and immediately visible oocytes) were counted. Magnification, 5×.



**Figure 2. Hypersensitivity to DNA Cross-linking Agents in Usp1-Deficient Cells**

(A) Increased Fancd2-Ub and PCNA-Ub in Usp1-deficient cells. Wild-type and *Usp1*<sup>-/-</sup> MEFs were left untreated, or were treated with MMC (500 ng/ml for 20 hr) or UV (30 J/m<sup>2</sup>, harvested at 3 hr after irradiation). Cell lysates were immunoblotted with indicated antibodies.

(B) Survival rates of wild-type and *Usp1*<sup>-/-</sup> MEFs to MMC and UV treatment are determined as the percentage of viable cells relative to that for respective untreated cells.

(C) MMC-induced chromosomal aberrations in wild-type (gray bars) and *Usp1*<sup>-/-</sup> (filled bars) MEFs following the treatment with MMC for 48 hr. The numbers of chromosomal aberrations (left panel) and radial forms (right panel) per metaphase spread were scored.

(D) Clonogenic survival assay of BM cells from wild-type (open squares), *Usp1*<sup>+/-</sup> (gray squares), *Usp1*<sup>-/-</sup> (filled squares), and *Fancd2*<sup>-/-</sup> (filled triangles) mice. BM cells were treated with increasing doses of MMC (left) or ionizing radiation (IR) (right). After 7–10 days in culture, the numbers of hematopoietic colonies were compared.

(E) Survival of wild-type (n = 10; gray squares) and *Usp1*<sup>-/-</sup> (n = 5; filled triangles) mice following 8.25 Gy of whole-body irradiation.

### FA Phenotype in Usp1-Deficient Lymphocytes

*Usp1*<sup>-/-</sup> bone marrow (BM) cells had increased monoubiquitination of Fancd2 and PCNA, as observed in *Usp1*<sup>-/-</sup> MEFs (data not shown). The comparison of the number of BM mononuclear cells

transformed human cell lines (Huang et al., 2006; Nijman et al., 2005a), *Usp1*<sup>-/-</sup> MEFs had a compensatory increase in total Fancd2 levels and in its monoubiquitination, as well as proliferating cell nuclear antigen (PCNA) monoubiquitination (Figure 2A). *Usp1*<sup>+/-</sup> MEFs exhibited an intermediate increase in Fancd2- and PCNA-monoubiquitination levels, suggesting a haploinsufficiency phenotype (Figure S3).

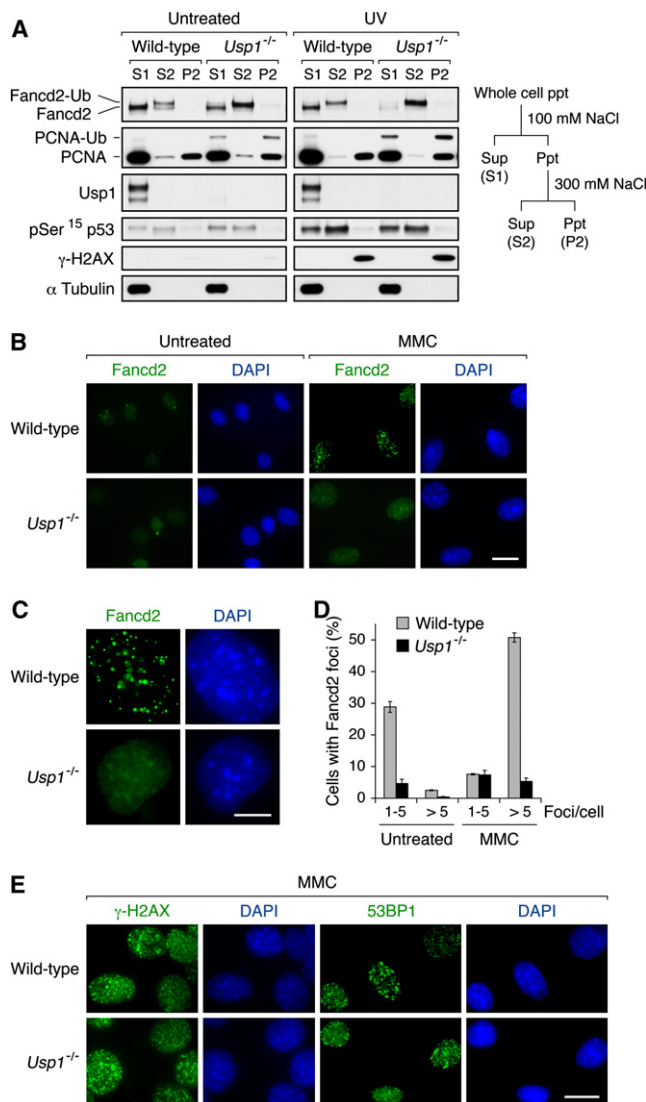
We next analyzed the response of *Usp1*<sup>-/-</sup> MEFs to DNA crosslinking agents. *Usp1*<sup>-/-</sup> MEFs showed elevated sensitivity to MMC treatment, whereas the sensitivity of *Usp1*<sup>-/-</sup> MEFs to ultraviolet (UV) irradiation was similar to that of wild-type cells (Figure 2B). Furthermore, following MMC treatment, *Usp1*<sup>-/-</sup> MEFs exhibited increased chromosomal breaks and radial forms that distinguish FA from other chromosomal breakage syndromes (Figure 2C). Retroviral transduction of wild-type Usp1 partially corrected the MMC-induced chromosome instability of *Usp1*<sup>-/-</sup> MEFs (Figure S2D). Since FA cells show an increased accumulation in G2/M following exposure to DNA interstrand crosslinkers, we examined whether Usp1-deficient cells show G2/M accumulation in response to MMC. After a short exposure of MMC, followed by recovery for 24 hr, *Usp1*<sup>-/-</sup> MEFs had an increase in G2/M content (57%) compared with that of wild-type MEFs (40%) (Figure S2C). Thus, *Usp1*<sup>-/-</sup> MEFs have a specific defect in DNA crosslink repair, similar to FA cells.

per hind limb indicated a significant reduction in *Usp1*<sup>-/-</sup> mice, whereas peripheral blood from *Usp1*<sup>-/-</sup> mice showed normal values for hemoglobin and white blood cell count (data not shown). Following methylcellulose culture in the presence of increasing concentrations of MMC, *Usp1*<sup>-/-</sup> BM cells, like *Fancd2*<sup>-/-</sup> BM cells, exhibited hypersensitivity to MMC compared with wild-type cells (Figure 2D). Both *Usp1*<sup>-/-</sup> and *Fancd2*<sup>-/-</sup> BM cells showed only mild sensitivity to IR compared with wild-type controls. However, Usp1-deficient mice displayed hypersensitivity to total body irradiation (Figure 2E), as has been described for Fancd2-deficient mice (Houghtaling et al., 2003).

### Increased Chromatin Accumulation of Monoubiquitinated Fancd2, but Decreased Nuclear Foci in Usp1-Deficient MEFs

Monoubiquitinated FANCD2 and FANCI are targeted to chromatin and form DNA repair foci in response to DNA damage (Garcia-Higuera et al., 2001; Smogorzewska et al., 2007). Since Usp1 depletion resulted in increased monoubiquitination of Fancd2, we examined whether this monoubiquitinated Fancd2 is localized to chromatin by using biochemical cell fractionation (Figure 3A). The "S1" fraction contains cytoplasmic and nucleoplasmic proteins; the "S2" fraction contains proteins bound to





**Figure 3. Increased Chromatin Accumulation of Monoubiquitinated Fancd2 and Impaired Fancd2 Foci Assembly in Usp1-Deficient MEFs** (A) Wild-type and *Usp1*<sup>-/-</sup> MEFs were either left untreated or treated with UV (30 J/m<sup>2</sup>). Cells were collected 3 hr after UV irradiation and fractionated. Each fraction was immunoblotted with the indicated antibodies. (B and C) Wild-type and *Usp1*<sup>-/-</sup> MEFs were stained for Fancd2 following either mock treatment (untreated) or 500 ng/ml of MMC for 20 hr. Scale bars, 10 μm (B) and 5 μm (C). (D) Quantification of cells with Fancd2 foci. Values are mean ± SEM, representing at least 700 nuclei each in three independent experiments. (E) Wild-type (upper panels) and *Usp1*<sup>-/-</sup> (lower panels) MEFs were stained for γ-H2AX and 53BP1 foci following MMC treatment (500 ng/ml) for 20 hr. Scale bar, 10 μm.

chromatin; and the “P2” fraction contains proteins tightly associated with nuclear structures (chromatin, nuclear matrix, etc. [Kim et al., 2008]). In *Usp1*<sup>-/-</sup> MEFs, monoubiquitinated Fancd2 efficiently localized in chromatin even in the absence of DNA damage (Figure 3A), suggesting that monoubiquitination is sufficient for chromatin targeting of Fancd2. A similar increase in chromatin-associated Fancd2 has been demonstrated in Usp1-depleted DT40 cells (Oestergaard et al., 2007).

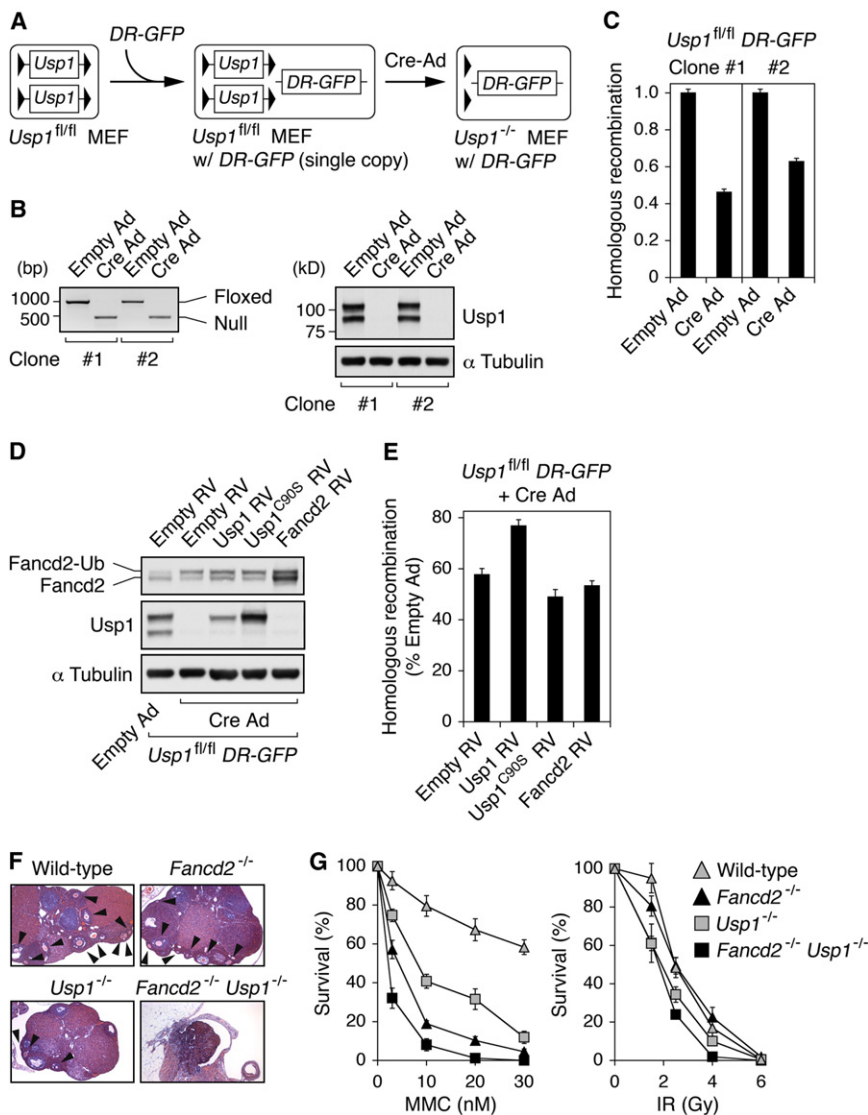
We next examined whether Fancd2 forms subnuclear foci in Usp1-deficient MEFs. Notably, *Usp1*<sup>-/-</sup> MEFs were defective in both spontaneous (S phase-specific) and DNA damage-inducible Fancd2 foci assembly (Figures 3B and 3C). Fancd2 foci formation was severely impaired in Usp1-deficient MEFs (Figure 3D), despite high levels of monoubiquitinated Fancd2 detected in the chromatin. The absence of Fancd2 foci therefore correlated with the MMC hypersensitivity of *Usp1*<sup>-/-</sup> cells. In contrast, Usp1-deficient MEFs exhibited normal levels of γ-H2AX foci and 53BP1 foci in response to MMC treatment (Figure 3E).

### HR Defect in Usp1-Deficient Cells

HR constitutes a central pathway to mediate repair of MMC-induced DNA damage (Niedzwiedz et al., 2004). Cells deficient for upstream or downstream FA proteins, including Fancd2, are deficient in HR (Litman et al., 2005; Nakanishi et al., 2005; Niedzwiedz et al., 2004; Sims et al., 2007; Smogorzewska et al., 2007; Xia et al., 2006; Yamamoto et al., 2005). The severe defect in Fancd2 foci formation in *Usp1*<sup>-/-</sup> MEFs raises the possibility of an HR defect. To determine whether Usp1 is required for HR, we generated *Usp1*<sup>fl/fl</sup> MEFs, in which each allele of *Usp1* is flanked by loxP sites (Figure 4A). We established four independent clones of *Usp1*<sup>fl/fl</sup> MEFs containing a single copy of the HR reporter DR-green fluorescent protein (GFP) (Pierce et al., 2001). These *Usp1*<sup>fl/fl</sup> DR-GFP clones were infected with adenovirus expressing Cre recombinase to generate *Usp1*<sup>-/-</sup> DR-GFP MEFs. Cre-mediated excision of the loxP-flanked *Usp1* gene was confirmed by PCR and immunoblotting (Figure 4B). Following the induction of HR by transfection with an I-SceI expression plasmid, *Usp1*<sup>-/-</sup> DR-GFP MEFs showed a 50% reduction in the frequency of GFP<sup>+</sup> cells compared with that of *Usp1*<sup>fl/fl</sup> DR-GFP MEFs (Figure 4C). Thus, the deficiency of Usp1 results in a defect in HR repair.

To confirm this effect in human cells, we used DR-U2OS cells, a human osteosarcoma cell line containing an integrated DR-GFP (Xia et al., 2006). siRNA against USP1 reduced the HR frequency in these cells to 45% of control, similar to the siRNA against FANCI (Figure S4).

The defect in HR observed in Usp1-deficient cells could occur because the cellular pool of Fancd2 is primarily ubiquitinated and bound to chromatin, leaving inadequate cellular levels of free Fancd2. If so, forced expression of Fancd2 could reverse the defect. To clarify this, *Usp1*<sup>fl/fl</sup> DR-GFP MEFs were stably transfected with empty retroviral vector, or the retroviral vector encoding wild-type Usp1, *Usp1*<sup>C90S</sup> (i.e., catalytically inactive form of the Usp1), or Fancd2, and these cells were subsequently infected with Cre-expressing adenovirus (Figures 4D and 4E). Expression of the heterologous proteins in the absence of endogenous Usp1 was confirmed by immunoblotting (Figure 4D). Immunoblotting for Fancd2 revealed a partially reverted L:S ratio (i.e., FANCD2-L [monoubiquitinated FANCD2] to FANCD2-S [unubiquitinated Fancd2] ratio) in *Usp1*<sup>-/-</sup> MEFs expressing wild-type Usp1, but not *Usp1*<sup>C90S</sup>. Consistent with this pattern, wild-type Usp1, but not *Usp1*<sup>C90S</sup>, corrected the HR defect of Usp1-deficient MEFs (Figure 4E). Interestingly, expression of excess Fancd2 did not restore HR activity, indicating that the HR defect in Usp1-deficient cells is not due to low cellular levels of free Fancd2.

**Figure 4. HR Defect in *Usp1*-Deficient MEFs**

(A) The scheme for the generation of *Usp1*<sup>-/-</sup> MEFs with DR-GFP reporter. Cre-Ad, Cre-expressing adenovirus.

(B) Confirmation of Cre-mediated excision of the loxP-flanked *Usp1* gene by genomic PCR (left panel) and Western blot (right panel). Empty-Ad, control adenovirus.

(C) Two independent *Usp1*<sup>fl/fl</sup> DR-GFP MEF clones were analyzed for HR frequencies, each with triplicates. Values were normalized for the transfection efficiency and are displayed as mean ± SEM GFP<sup>+</sup> frequencies relative to that of each *Usp1*<sup>fl/fl</sup> DR-GFP clone.

(D) Immunoblotting of *Usp1*<sup>fl/fl</sup> DR-GFP MEFs expressing wild-type *Usp1*, *Usp1*<sup>C90S</sup>, or *Fancd2* following Cre-expressing adenovirus infection.

(E) I-SceI-induced HR frequencies in *Usp1*<sup>-/-</sup> DR-GFP MEFs transduced with empty retroviral vector or the retroviral vector encoding wild-type *Usp1*, *Usp1*<sup>C90S</sup>, or *Fancd2*. Values were normalized for the transfection efficiency and are displayed as mean ± SEM GFP<sup>+</sup> frequencies relative to that of each *Usp1*<sup>fl/fl</sup> DR-GFP MEF.

(F) Histopathology of ovaries from 12-week-old wild-type, *Fancd2*<sup>-/-</sup>, *Usp1*<sup>-/-</sup>, and *Fancd2*<sup>-/-</sup> *Usp1*<sup>-/-</sup> siblings generated by *Usp1*<sup>+/-</sup> *Fancd2*<sup>+/-</sup> crosses. *Fancd2*<sup>-/-</sup> *Usp1*<sup>-/-</sup> double-knockout females had a more severe ovarian atrophy than either group of single-knockout mice. Magnification, 5×. Arrowheads, oocytes.

(G) Survival rates of BM cells from wild-type (gray triangles), *Usp1*<sup>-/-</sup> (gray squares), *Fancd2*<sup>-/-</sup> (filled triangles), and *Fancd2*<sup>-/-</sup> *Usp1*<sup>-/-</sup> (filled squares) mice following treatment with increasing doses of MMC (left panel) or IR (right panel). After 7–10 days in culture, the numbers of hematopoietic colonies were compared.

### Double Knockout of *Usp1* and *Fancd2* Results in a More Severe Phenotype

We next generated *Usp1*<sup>-/-</sup> *Fancd2*<sup>-/-</sup> double-knockout mice by crossing *Usp1*<sup>+/-</sup> *Fancd2*<sup>+/-</sup> mice. Interestingly, *Usp1*<sup>-/-</sup> *Fancd2*<sup>-/-</sup> females showed more severe ovarian atrophy than either group of single-knockout mice (Figure 4F). Western blots showed that DNA damage signaling in response to MMC or UV irradiation is not significantly different from that of either of the single mutants or wild-type controls (Figure S5). However, an increase in baseline phosphorylation of Chk2 was observed in *Usp1*<sup>-/-</sup> *Fancd2*<sup>-/-</sup> fibroblasts, suggesting that spontaneous DNA damage may accumulate in *Usp1*<sup>-/-</sup> *Fancd2*<sup>-/-</sup> mice (Figure S5). Furthermore, BM cells from *Usp1*<sup>-/-</sup> *Fancd2*<sup>-/-</sup> mice were more sensitive to MMC than either group of single-knockout mice (Figure 4G). Although it has been reported that mice deficient in Fanc proteins tend to display more severe phenotype in C57B6 background (AgoulNIK et al., 2002; Chen et al., 1996; Houghtaling et al., 2003; Wong et al., 2003), our *Usp1*<sup>-/-</sup> *Fancd2*<sup>-/-</sup> mice in C57BL/6 × 129Sv mixed genetic

background consistently showed enhanced defects compared with either their single-knockout littermates or *Usp1*<sup>-/-</sup> mice in C57B6 background (Figure 4G and data not shown). Therefore, it is unlikely that the severe phenotype observed for *Usp1*<sup>-/-</sup> *Fancd2*<sup>-/-</sup> mice is solely due to the difference in genetic background. Taken together, these data suggest that *Usp1* may regulate additional DNA repair pathways in addition to the FA pathway, perhaps by controlling the ubiquitination state of substrates other than *Fancd2* or *Fanci*.

### DISCUSSION

We have demonstrated that *Usp1*-deficient mice have a similar phenotype to that of other FA mouse models. FA-deficient mice are generally small, exhibit reduced fertility, and have heightened crosslinker sensitivity of their primary cells (AgoulNIK et al., 2002; Chen et al., 1996; Cheung et al., 2004; Houghtaling et al., 2003; Koomen et al., 2002; McAllister et al., 2002; Pellas et al., 1991; Whitney et al., 1996; Wong et al., 2003;

Yang et al., 2001). Usp1-deficient mice are small and exhibit male infertility, chromosomal instability, and cellular hypersensitivity to crosslinking agents. Moreover, the Usp1-deficient mice exhibit a more severe phenotype than most FA mouse models, with approximately 80% perinatal lethality, testicular atrophy, and depletion of male germ cells. Such a similarity in phenotypes of mice deficient in *Usp1* and *Fanc* genes further argues for their genetic (epistatic) relationships. At the same time, the elevated perinatal lethality and heightened cellular levels of PCNA-Ub in *Usp1*<sup>-/-</sup> cells, which are not observed in FA cells, suggest that Usp1 may have other functions (and perhaps other substrates) in somatic cells, which may account, at least in part, for the absence of USP1 deficiency in humans.

The mechanism by which USP1 depletion leads to MMC hypersensitivity is unknown, and several models are possible. First, loss of USP1 results in elevated FANCD2-Ub and depletion of the unubiquitinated form of FANCD2 (FANCD2-S). Loss of FANCD2-S may impair the ability of a cell to respond to additional crosslink damage. Accordingly, the normal function of USP1 may be to recycle free FANCD2-S (and free Ubiquitin) in the cell. However, forced expression of *Fancd2* in *Usp1*<sup>-/-</sup> MEFs did not correct the HR defect (Figures 4A–4C). Alternatively, excessive chromatin accumulation of *Fancd2*-Ub in *Usp1*<sup>-/-</sup> cells may recruit an additional repair factor to chromatin, reducing its availability to participate at sites of damage. Second, elevated *Fancd2*-Ub levels in the Usp1-deficient MEFs may be toxic to cells, and promote MMC sensitivity. However, double knockout of *Usp1* and *Fancd2* in mice did not improve the MMC sensitivity, suggesting that high levels of FANCD2 ubiquitination do not account for the cellular phenotype of *Usp1*<sup>-/-</sup> mice (Figures 4F and G). Third, coupled ubiquitination and deubiquitination may be necessary for proper FA pathway function, suggesting that USP1 acts at a critical later step in the FA pathway itself (Figure S6). For instance, FANCD2 monoubiquitination by the FA core complex promotes chromatin loading and assembly of DNA repair complexes. USP1-mediated deubiquitination of FANCD2-Ub may catalyze additional later events in the DNA repair process. Consistent with this model, Usp1-deficient cells have elevated *Fancd2*-Ub in chromatin, but impaired foci formation of *Fancd2*.

Usp1 depletion also results in prolonged, elevated levels of PCNA-Ub. Depletion of USP1 in human cell lines, by siRNA, results in elevated PCNA-Ub levels and elevated mutagenesis (Huang et al., 2006), secondary to increased recruitment of error-prone polymerases, such as DNA polymerase  $\eta$ . One might expect, therefore, that Usp1-deficient mice will exhibit an increased point mutation frequency and, perhaps, an increased cancer incidence.

## EXPERIMENTAL PROCEDURES

### Generation of Usp1 Knockout Mice

Usp1 conditional knockout mice (in C57BL/6J genetic background) were generated by Ingenko under a consortium agreement. A targeting vector was engineered to disrupt the *Usp1* gene in C57BL/6J-derived embryonic stem cells. Exon 3 was targeted for replacement by exon 3 flanked by *loxP* sites and *neo*<sup>r</sup> cassette flanked by Flp recombination target sites. Ella-Cre-transgenic female mice (JAX stock no. 003724) were used to generate heterozygous *Usp1*<sup>+/-Cre</sup> mutant mice. The Cre transgene was removed by backcrossing to wild-type C57BL/6J mice.

*Fancd2* knockout mice (C57BL/6 × 129/Sv mixed genetic background) were generated by a gene-trap method (K. Parmar and A. D'Andrea, unpublished data), and these mice had a phenotype similar to that observed in a previous report (Houghtaling et al., 2003). Mice deficient for both *Usp1* and *Fancd2* were generated by intercrossing of *Usp1*<sup>+/-</sup>*Fancd2*<sup>+/-</sup> mice.

### HR Assay

For HR assay with MEFs, the DR-GFP reporter, hypoxanthine-guanine phosphoribosyl transferase-DR-GFP-hygromycin (Nakanishi et al., 2005), was integrated into the genome of *Usp1*<sup>fl/fl</sup> MEFs. Clones with a single copy of DR-GFP were identified by Southern blotting. These clones were then used for infection with either an “empty” pBABE-puro retrovirus or the pBABE-puro retrovirus into which wild-type Usp1, Usp1<sup>C90S</sup>, or *Fancd2* cDNA was inserted. Retrovirus-infected cells were selected in puromycin before infection with adenovirus expressing Cre recombinase. *Usp1*<sup>fl/fl</sup> DR-GFP MEFs were transfected with I-SceI expression plasmid (pCBASce) in triplicate wells of 24-well plates using Lipofectamine 2000. The parallel transfection with pEGFP-C1 was used to normalize transfection efficiency. At 48 hr after transfection, cells were trypsinized and single-cell suspensions were analyzed by flow cytometry.

### SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at [http://www.developmentalcell.com/supplemental/S1534-5807\(09\)00003-3/](http://www.developmentalcell.com/supplemental/S1534-5807(09)00003-3/).

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### REFERENCES

- AgoulNIK, A.I., Lu, B., Zhu, Q., Truong, C., Ty, M.T., Arango, N., Chada, K.K., and Bishop, C.E. (2002). A novel gene, *Pog*, is necessary for primordial germ cell proliferation in the mouse and underlies the germ cell deficient mutation, *gcd*. *Hum. Mol. Genet.* 11, 3047–3053.
- Chen, M., Tomkins, D.J., Auerbach, W., McKelvie, C., Youssoufian, H., Liu, L., Gan, O., Carreau, M., Auerbach, A., Groves, T., et al. (1996). Inactivation of *Fac* in mice produces inducible chromosomal instability and reduced fertility reminiscent of Fanconi anaemia. *Nat. Genet.* 12, 448–451.
- Cheung, A.M., Elia, A., Tsao, M.S., Done, S., Wagner, K.U., Hennighausen, L., Hakem, R., and Mak, T.W. (2004). *Brca2* deficiency does not impair mammary epithelium development but promotes mammary adenocarcinoma formation in *p53*<sup>+/-</sup> mutant mice. *Cancer Res.* 64, 1959–1965.
- Cohn, M.A., Kowal, P., Yang, K., Haas, W., Huang, T.T., Gygi, S.P., and D'Andrea, A.D. (2007). A UAF1-containing multisubunit protein complex regulates the Fanconi anemia pathway. *Mol. Cell* 28, 786–797.
- Dorsman, J.C., Levitus, M., Rockx, D., Rooimans, M.A., Oostra, A.B., Haitjema, A., Bakker, S.T., Steltenpool, J., Schuler, D., Mohan, S., et al. (2007). Identification of the Fanconi anemia complementation group I gene, *FANCI*. *Cell. Oncol.* 29, 211–218.
- Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M.S., Timmers, C., Hejna, J., Grompe, M., and D'Andrea, A.D. (2001). Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol. Cell* 7, 249–262.

- Grompe, M., and van de Vrugt, H. (2007). The Fanconi family adds a fraternal twin. *Dev. Cell* 12, 661–662.
- Houghtaling, S., Timmers, C., Noll, M., Finegold, M.J., Jones, S.N., Meyn, M.S., and Grompe, M. (2003). Epithelial cancer in Fanconi anemia complementation group D2 (*Fancc2*) knockout mice. *Genes Dev.* 17, 2021–2035.
- Huang, T.T., Nijman, S.M., Mirchandani, K.D., Galardy, P.J., Cohn, M.A., Haas, W., Gygi, S.P., Ploegh, H.L., Bernards, R., and D'Andrea, A.D. (2006). Regulation of monoubiquitinated PCNA by DUB autocleavage. *Nat. Cell Biol.* 8, 339–347.
- Kim, J.M., Kee, Y., Gurtan, A., and D'Andrea, A.D. (2008). Cell cycle-dependent chromatin loading of the Fanconi anemia core complex by FANCM/FAAP24. *Blood* 111, 5215–5222.
- Koomen, M., Cheng, N.C., van De Vrugt, H.J., Godthelp, B.C., van Der Valk, M.A., Oostra, A.B., Zdzienicka, M.Z., Joenje, H., and Arwert, F. (2002). Reduced fertility and hypersensitivity to mitomycin C characterize *Fancg/Xrcc9* null mice. *Hum. Mol. Genet.* 11, 273–281.
- Litman, R., Peng, M., Jin, Z., Zhang, F., Zhang, J., Powell, S., Andreassen, P.R., and Cantor, S.B. (2005). BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product FANCD2. *Cancer Cell* 8, 255–265.
- McAllister, K.A., Bennett, L.M., Houle, C.D., Ward, T., Malphurs, J., Collins, N.K., Cachafeiro, C., Haseman, J., Goulding, E.H., Bunch, D., et al. (2002). Cancer susceptibility of mice with a homozygous deletion in the COOH-terminal domain of the *Brca2* gene. *Cancer Res.* 62, 990–994.
- Montes De Oca, R., Andreassen, P.R., Margossian, S.P., Gregory, R.G., Taniguchi, T., Wang, X., Houghtaling, S., Grompe, M., and D'Andrea, A.D. (2004). Regulated interaction of the Fanconi anemia protein, FANCD2, with chromatin. *Blood* 105, 1003–1009.
- Nakanishi, K., Yang, Y.G., Pierce, A.J., Taniguchi, T., Digweed, M., D'Andrea, A.D., Wang, Z.Q., and Jasin, M. (2005). Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. *Proc. Natl. Acad. Sci. USA* 102, 1110–1115.
- Niedzwiedz, W., Mosedale, G., Johnson, M., Ong, C.Y., Pace, P., and Patel, K.J. (2004). The Fanconi anaemia gene *FANCC* promotes homologous recombination and error-prone DNA repair. *Mol. Cell* 15, 607–620.
- Nijman, S.M., Huang, T.T., Dirac, A.M., Brummelkamp, T.R., Kerkhoven, R.M., D'Andrea, A.D., and Bernards, R. (2005a). The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. *Mol. Cell* 17, 331–339.
- Nijman, S.M., Luna-Vargas, M.P., Velds, A., Brummelkamp, T.R., Dirac, A.M., Sixma, T.K., and Bernards, R. (2005b). A genomic and functional inventory of deubiquitinating enzymes. *Cell* 123, 773–786.
- Oestergaard, V.H., Langevin, F., Kuiken, H.J., Pace, P., Niedzwiedz, W., Simpson, L.J., Ohzeki, M., Takata, M., Sale, J.E., and Patel, K.J. (2007). Deubiquitination of FANCD2 is required for DNA crosslink repair. *Mol. Cell* 28, 798–809.
- Pellas, T.C., Ramachandran, B., Duncan, M., Pan, S.S., Marone, M., and Chada, K. (1991). Germ-cell deficient (*gcd*), an insertional mutation manifested as infertility in transgenic mice. *Proc. Natl. Acad. Sci. USA* 88, 8787–8791.
- Pierce, A.J., Hu, P., Han, M., Ellis, N., and Jasin, M. (2001). Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells. *Genes Dev.* 15, 3237–3242.
- Sims, A.E., Spiteri, E., Sims, R.J., 3rd, Arita, A.G., Lach, F.P., Landers, T., Wurm, M., Freund, M., Neveling, K., Hanenberg, H., et al. (2007). FANCI is a second monoubiquitinated member of the Fanconi anemia pathway. *Nat. Struct. Mol. Biol.* 14, 564–567.
- Smogorzewska, A., Matsuo, S., Vinciguerra, P., McDonald, E.R., 3rd, Hurov, K.E., Luo, J., Ballif, B.A., Gygi, S.P., Hofmann, K., D'Andrea, A.D., and Elledge, S.J. (2007). Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. *Cell* 129, 289–301.
- Wang, W. (2007). Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. *Nat. Rev. Genet.* 8, 735–748.
- Whitney, M.A., Royle, G., Low, M.J., Kelly, M.A., Axthelm, M.K., Reifsteck, C., Olson, S., Braun, R.E., Heinrich, M.C., Rathbun, R.K., et al. (1996). Germ cell defects and hematopoietic hypersensitivity to gamma-interferon in mice with a targeted disruption of the Fanconi anemia C gene. *Blood* 88, 49–58.
- Wong, J.C., Alon, N., McKerlie, C., Huang, J.R., Meyn, M.S., and Buchwald, M. (2003). Targeted disruption of exons 1 to 6 of the Fanconi anemia group A gene leads to growth retardation, strain-specific microphthalmia, meiotic defects and primordial germ cell hypoplasia. *Hum. Mol. Genet.* 12, 2063–2076.
- Xia, B., Sheng, Q., Nakanishi, K., Ohashi, A., Wu, J., Christ, N., Liu, X., Jasin, M., Couch, F.J., and Livingston, D.M. (2006). Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. *Mol. Cell* 22, 719–729.
- Yamamoto, K., Hirano, S., Ishiai, M., Morishima, K., Kitao, H., Namikoshi, K., Kimura, M., Matsushita, N., Arakawa, H., Buerstedde, J.M., et al. (2005). Fanconi anemia protein FANCD2 promotes immunoglobulin gene conversion and DNA repair through a mechanism related to homologous recombination. *Mol. Cell Biol.* 25, 34–43.
- Yang, Y., Kuang, Y., De Oca, R.M., Hays, T., Moreau, L., Lu, N., Seed, B., and D'Andrea, A.D. (2001). Targeted disruption of the murine Fanconi anemia gene, *Fancg/Xrcc9*. *Blood* 98, 3435–3440.